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ORIGINAL ARTICLE

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$^{15}N/^{14}N$ ratios of amino acids as a tool for studying terrestrial food webs: a case study of terrestrial insects (bees, wasps, and hornets)

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Abstract Compound-specific stable isotope analysis (CSIA) of amino acids is a new method that enables estimates of trophic position for consumers in food webs. We examined the nitrogen isotopic composition ($\delta^{15}N$) of amino acids of Japanese social insects (three bee, three wasp, and four hornet species) to evaluate the potential of CSIA of amino acids in studies of terrestrial food webs. For wasps, we also examined samples at different growth stages (ranging from egg to adult) to assess the effect of metamorphosis on CSIA estimates of trophic position. The $\delta^{15}N$ values of bulk tissues for Japanese social insects are only weakly correlated with the biologically expected trophic positions. In contrast, the trophic positions estimated from the $\delta^{15}N$ values of amino acids (yielding values of between 2.0 and 2.3 for bees, between 2.8 and 3.3 for wasps, and between 3.5 and 4.1 for hornets) are consistent with the biologically expected trophic positions for these insects (i.e., 2.0 for bees, 3.0 for wasps, and 3.0–4.0 for hornets). Although large variability is observed among the $\delta^{15}N$ values of individual amino acids (e.g., ranging from 3.0 to 14.9‰ for phenylalanine), no significant change is observed in the trophic position during wasp metamorphosis. Thus, the CSIA of amino acids is a powerful tool for investigating not only aquatic food webs but also terrestrial food webs with predatory insects.

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Introduction

Knowledge of the trophic position of organisms in food webs allows understanding of biomass flow and trophic linkages in complex networks of ecosystems. Compound-specific stable isotope analysis (CSIA) of amino acids is a new method that enables estimates of the trophic position of organisms in food webs (e.g., McClelland and Montoya 2002; Popp et al. 2007; Chikaraishi et al. 2009). It has been proposed that a comparison between large ^{15}N-enrichment (+8.0‰) in glutamic acid and little change (+0.4‰) in phenylalanine, with each increase in trophic level, provides a precise estimate of the trophic position of organisms in food webs (Fig. 1). This approach is based on isotopic fractionations associated with the metabolic processes of two common amino acids: glutamic acid shows significant increase of $\delta^{15}N$ values during reactions (e.g., transamination and deamination) that cleaves the carbon–nitrogen bond, whereas phenylalanine shows little change in $\delta^{15}N$ values during conversion to tyrosine that neither forms nor cleaves the carbon–nitrogen bond (Chikaraishi et al. 2007). Chikaraishi et al. (2009, 2010a) established that the trophic position ($TP_{Glu/Phe}$) of organisms could be estimated from the nitrogen isotopic compositions of glutamic acid ($\delta^{15}N_{Glu}$) and phenylalanine ($\delta^{15}N_{Phe}$), as follows:

$$TP_{Glu/Phe} = [(\delta^{15}N_{Glu} - \delta^{15}N_{Phe} + \beta)/7.6] + 1 \quad (1)$$

where β represents the isotopic difference between glutamic acid and phenylalanine in primary producers (−3.4‰ for aquatic cyanobacteria and algae, +8.4‰ for terrestrial C3 plants, and −0.4‰ for terrestrial C4 plants; Fig. 1). This method has three key advantages. First, the trophic position is estimated based on the $\delta^{15}N$ values of amino acids only from the target

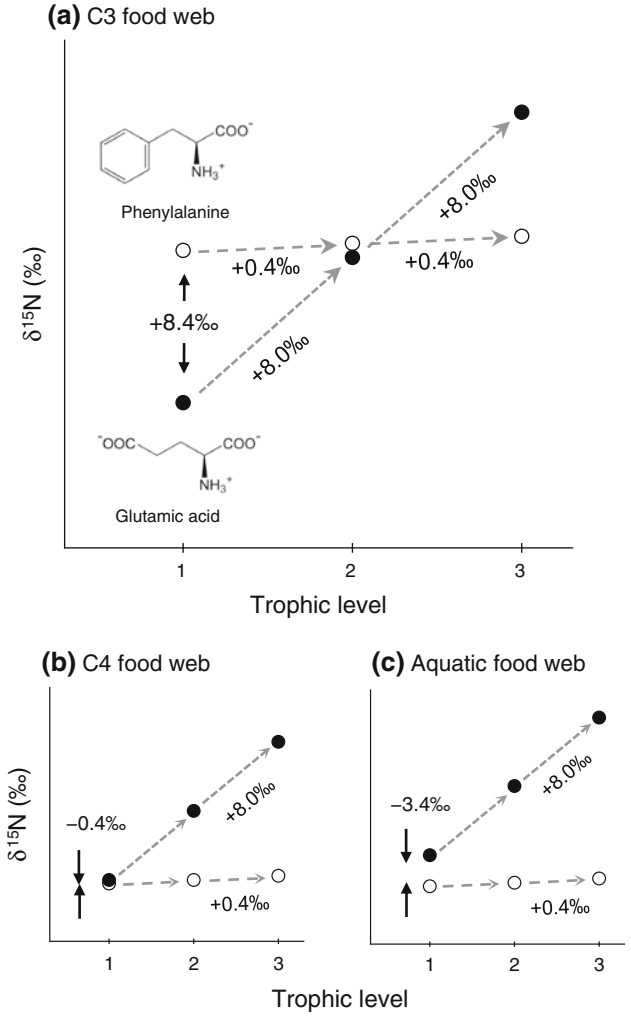

Fig. 1 Schematic illustration of the relationship between the nitrogen isotopic composition of amino acids (glutamic acid and phenylalanine) and trophic level in food webs (after Chikaraishi et al. 2009, 2010a)

organism. Consequently, unlike bulk isotopic methods (i.e., determination of the trophic position from bulk nitrogen isotope analysis; e.g., Minagawa and Wada 1984), this method does not require characterization of the δ^{15}N values of primary producers or baseline consumers. Second, small accuracy is expected; the standard deviation (1σ) of accuracy in the trophic position ($=$[actual TP] $-$ [$TP_{Glu/Phe}$]) is only 0.12 units for aquatic organisms (Chikaraishi et al. 2009) and 0.20 units for terrestrial organisms (Chikaraishi et al. 2010a). Third, a small sample size (nanomolar levels of nitrogen) is required for CSIA of amino acids by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) (Merritt and Hayes 1994; Chikaraishi et al. 2010b). As a result, CSIA of amino acids overcomes several of the challenges associated with bulk isotopic methods (e.g., O'Reilly et al. 2002; Post 2002). Consequently, the CSIA of amino acids has been used to elucidate the trophic position of organisms in recent ecological studies of shrimp in the sub-Antarctic archipelago (Pakhomov et al. 2004), krill in the Antarctic (Schmidt et al. 2004, 2006), plankton in the central Pacific (McCarthy et al. 2007), yellow-fin tuna in the eastern tropical Pacific (Popp et al. 2007), zooplankton near Hawaii (Hannides et al. 2009), and penguin in the Southern Ocean (Lorrain et al. 2009).

Recent evidence suggests that CSIA of amino acids is also useful in the evaluation of terrestrial food webs (Chikaraishi et al. 2010a; Naito et al. 2010; Styring et al. 2010). In particular, Chikaraishi et al. (2010a) demonstrated that a ^{15}N enrichment factor of $+7.6‰$ (Eq. 1) describes the trophic relationship between caterpillars and plant leaves (i.e., diet), which is consistent with relationship found between aquatic organisms and their diets (Chikaraishi et al. 2009). However, few studies have used CSIA of amino acids to evaluate terrestrial food webs. In terrestrial ecosystems, insects are abundant, and are the dominant consumers at many trophic levels (e.g., Price et al. 1980). However, the factors controlling the isotopic composition of amino acids and the tropic position of insects remain poorly understood. In particular, many insects undergo a metamorphosis from larval to adult stages. Little information is available regarding isotopic and trophic changes associated with metamorphosis, from feeding during the larval and adult stages through starvation during the chrysalis stage. Also, pollen and nectar are basal resources in terrestrial food webs (e.g., Memmott 1999; O'Reilly et al. 2002, Patt et al. 2003), and feeding on pollen and nectar by pollinators is important for maintaining plant–pollinator interactions and terrestrial food webs (Memmott 1999). However, investigations of feeding on pollen and nectar would be difficult using only bulk δ^{15}N values (Patt et al. 2003), because large variations exist among the δ^{15}N values of these foods.

To further evaluate the applicability of CSIA of amino acids for food webs in terrestrial ecosystems, we examined the nitrogen isotopic compositions of amino acids in 45 samples of social insects, including three bee, three wasp, and four hornet species, as well as in 20 samples of reference materials including plant, caterpillar, aphid, and ladybug samples. For the three wasp species, we examined 26 samples, which included wasps at different growth stages (from egg to adult), which were used to evaluate the effect, if any, of metamorphosis on estimates of trophic position.

Materials and methods

All samples were collected from a farm near Yokohama, Japan (35°08′N, 139°07′E) (Table 1). This farm cultivates fruits (e.g., oranges and chestnuts) and vegetables (e.g., carrots, broccoli, eggplants, potatoes, radishes, and tomatoes), and all are C3 plants. Samples of adult bees (*Apis mellifera*, *Bombus diversus diversus,* and *Xylocopa appendiculata circumvolans*), wasps (*Polistes japonicus japonicus*, *Polistes rothneyi iwatai*, and *Parapolybia indica*), and hornets (*Vespa ducalis pulchra*, *Vespa*

Table 1 Nitrogen isotopic composition of 10 amino acids in bee, wasp, hornet, and reference materials (plant, caterpillar, aphid, and ladybug) used in this study

Sample	Stage	Tissue	$\delta^{13}C_{Bulk}$	$\delta^{15}N$											TP^{a}_{bulk}	$TP^{b}_{Glu/Phe}$
				Bulk	Alanine	Glycine	Valine	Leucine	Isolucine	Proline	Serine	Methionine	Glutamic acid	Phenyl -alanine		
Bee																
Apis mellifera (#1)	Adult	Whole	−26.8	1.6	4.8	6.5	6.4	0.9	3.2	15.0	2.8	1.4	8.0	9.1	0.5	2.0
Apis mellifera (#2)	Adult	Whole	n.d.	n.d.	4.1	3.4	3.2	−0.2	0.2	10.1	0.9	n.d.	7.3	7.2	n.d.	2.1
Apis mellifera (#3)	Adult	Whole	n.d.	n.d.	6.3	6.9	7.5	5.8	5.5	20.8	n.d.	3.0	11.7	11.6	n.d.	2.1
Bombus diversus diversus	Adult	Whole	−26.9	2.2	2.4	2.1	2.0	−0.8	0.3	12.2	n.d.	−0.7	6.9	5.7	0.7	2.3
Xylocopa appendiculata circumvolans	Adult	Whole	−25.0	5.1	9.8	10.3	11.1	11.5	7.8	19.1	6.0	−1.1	12.3	12.6	1.5	2.1
Wasp																
Polistes japonicus japonicus (#X)	Egg	Whole	−27.2	4.9	7.9	−1.0	16.4	7.6	10.0	16.7	−1.6	0.0	19.9	14.1	1.5	2.9
Polistes japonicus japonicus (#2)	Larva	Whole	−29.7	1.6	4.2	2.1	15.7	2.1	5.1	14.0	0.0	−1.5	16.8	9.9	0.5	3.0
Polistes japonicus japonicus (#3)	Larva	Whole	−30.1	1.1	4.6	1.0	14.4	1.4	5.0	13.6	−0.6	−3.4	17.3	8.7	0.3	3.2
Polistes japonicus japonicus (#4)	Chrysalis	Whole	−29.4	1.4	3.6	1.5	16.4	5.2	7.4	17.5	−0.5	n.d.	17.3	10.2	0.4	3.0
Polistes japonicus japonicus (#5)	Chrysalis	Whole	−28.6	2.6	8.8	2.2	15.6	3.7	5.2	15.9	−0.5	n.d.	18.6	11.2	0.8	3.1
Polistes japonicus japonicus (#6)	Newly emerged	Whole	−28.8	2.7	9.0	1.2	14.0	8.1	8.9	17.0	−4.1	−0.4	17.7	12.0	0.8	2.9
Polistes rothneyi iwatai (#1)	Egg	Whole	−26.1	7.2	9.0	5.1	16.2	10.6	13.5	18.2	10.1	n.d.	22.5	13.2	2.1	3.3
Polistes rothneyi iwatai (#2)	Larva	Whole	−27.4	4.6	7.4	2.7	14.3	8.2	12.3	16.3	4.3	n.d.	20.7	13.7	1.4	3.0
Polistes rothneyi iwatai (#3)	Larva	Whole	−27.2	5.5	8.0	0.9	16.0	9.8	13.6	17.9	1.2	n.d.	20.9	13.5	1.6	3.1
Polistes rothneyi iwatai (#4)	Larva	Whole	−27.2	5.2	8.3	5.5	15.3	9.0	13.9	18.4	5.8	n.d.	19.8	13.5	1.5	2.9
Polistes rothneyi iwatai (#5)	Chrysalis	Whole	−29.6	5.5	7.3	2.7	14.3	10.6	12.2	17.3	−0.3	n.d.	20.3	12.9	1.6	3.1
Polistes rothneyi iwatai (#6)	Chrysalis	Whole	−28.6	5.5	6.0	3.3	15.1	10.0	14.2	18.1	0.8	n.d.	19.5	12.0	1.6	3.1
Polistes rothneyi iwatai (#7)	Chrysalis	Whole	−29.8	5.4	8.6	3.2	14.0	10.9	13.7	18.4	3.8	n.d.	20.5	13.6	1.6	3.0
Polistes rothneyi iwatai (#8)	Chrysalis	Whole	−28.1	4.9	9.5	8.5	15.9	12.0	15.3	16.5	1.6	n.d.	21.1	14.4	1.5	3.0
Polistes rothneyi iwatai (#9)	Chrysalis	Whole	−28.4	4.9	7.8	6.4	15.6	8.4	13.8	18.2	4.7	n.d.	19.6	13.5	1.5	2.9
Polistes rothneyi iwatai (#10)	Chrysalis	Whole	−28.2	4.9	6.4	10.0	16.7	12.5	15.6	16.7	0.5	n.d.	22.0	14.9	1.5	3.0
Polistes rothneyi iwatai (#11)	Newly emerged	Whole	−28.4	2.4	5.5	1.2	11.7	9.7	10.9	14.2	−0.6	n.d.	14.1	8.3	0.7	2.9
Polistes rothneyi iwatai (#12)	Adult	Whole	−26.8	5.5	12.2	8.4	9.0	5.6	5.6	n.d.	3.0	5.7	11.3	6.2	1.6	2.8
Polistes rothneyi iwatai (#13)	Adult	Whole	n.d.	n.d.	6.1	5.5	8.8	7.5	11.0	12.6	4.0	−1.8	14.3	6.1	n.d.	3.2
Polistes rothneyi iwatai (#14)	Adult	Whole	n.d.	n.d.	5.9	4.5	8.6	5.6	8.6	13.6	3.0	2.7	15.9	8.4	n.d.	3.1
Polistes rothneyi iwatai (#15-1)	Newly emerged	Abdomen	−29.0	3.8	13.0	5.7	16.9	11.1	14.6	19.2	6.6	n.d.	21.4	14.8	1.1	3.0
Polistes rothneyi iwatai (#15-2)	Newly emerged	Head	−28.0	6.4	12.4	6.6	16.9	11.4	14.4	19.7	6.5	n.d.	21.3	14.1	1.9	3.1
Polistes rothneyi iwatai (#15-3)	Newly emerged	Leg	−28.6	5.4	12.9	5.4	16.3	11.8	14.2	19.2	6.6	n.d.	20.8	14.3	1.6	3.0
Polistes rothneyi iwatai (#15-4)	Newly emerged	Thorax	−28.4	5.7	12.9	6.1	16.4	10.6	13.7	19.6	n.d.	n.d.	20.9	14.2	1.7	3.0
Polistes rothneyi iwatai (#15-5)	Newly emerged	Wing	−27.6	5.4	12.9	6.5	16.2	12.4	13.8	20.2	7.3	n.d.	21.7	14.9	1.6	3.0
Parapolybia indica (#1)	Larva	Whole	−27.7	3.5	6.5	4.9	9.5	6.6	8.2	15.9	3.5	n.d.	14.9	9.1	1.0	2.9
Parapolybia indica (#2)	Larva	Whole	−28.5	2.5	7.6	7.5	11.6	3.5	6.3	15.7	2.8	n.d.	13.8	6.3	0.8	3.1
Parapolybia indica (#3)	Larva	Whole	−28.6	1.3	7.6	8.6	10.5	4.5	5.5	14.0	2.5	n.d.	12.0	5.1	0.4	3.0
Parapolybia indica (#4)	Chrysalis	Whole	−28.4	3.7	8.0	8.4	11.1	6.0	9.6	16.2	2.6	n.d.	16.8	11.2	1.1	2.8
Parapolybia indica (#5)	Chrysalis	Whole	−28.3	4.0	8.2	7.9	12.2	4.7	6.7	15.9	1.3	n.d.	12.7	4.8	1.2	3.1
Parapolybia indica (#6)	Chrysalis	Whole	−27.6	3.3	7.6	5.8	11.4	5.9	7.2	13.9	3.4	n.d.	9.6	3.0	1.0	3.0

Table 1 continued

Sample	Stage	Tissue	$\delta^{13}C_{Bulk}$	$\delta^{15}N$										TP^{a}_{bulk}	$TP^{b}_{Glu/Phe}$
				Bulk	Alanine	Glycine	Valine	Leucine	Isolucine	Proline	Serine	Methionine	Glutamic acid	Phenyl -alanine	
Parapolybia indica (#7)	Chrysalis	Whole	−28.7	1.4	6.9	5.6	10.0	6.9	7.2	19.5	7.7	n.d.	10.0	4.4	0.4	2.8
Parapolybia indica (#8)	Newly emerged	Whole	−27.4	4.9	6.0	2.7	9.8	9.5	9.1	15.5	2.6	n.d.	14.9	8.2	1.5	3.0
Parapolybia indica (#9)	Adult	Whole	−27.5	4.3	7.8	6.2	10.0	6.9	8.4	15.9	5.4	n.d.	15.6	8.8	1.3	3.0
Hornet																
Vespa ducalis pulchra (#1)	Adult	Whole	−25.8	5.4	7.6	5.4	19.9	10.7	13.2	19.1	4.5	n.d.	21.4	7.7	1.6	3.9
Vespa ducalis pulchra (#2)	Adult	Whole	n.d.	n.d.	10.7	9.5	17.2	10.1	13.2	20.6	6.8	n.d.	23.5	8.8	n.d.	4.0
Vespa ducalis pulchra (#3)	Adult	Whole	n.d.	n.d.	10.0	4.5	14.8	11.0	15.5	16.1	3.0		22.6	7.4	n.d.	4.1
Vespa mandarinia japonica	Adult	Whole	−26.8	4.5	13.2	−0.2	16.8	6.6	8.5	15.3	0.6	1.0	18.5	8.0	1.3	3.5
Vespa simillima xanthoptera	Adult	Whole	−25.6	4.9	12.0	7.0	12.2	7.2	10.2	18.3	3.6	n.d.	20.1	9.4	1.5	3.5
Vespula flaviceps lewisii	Adult	Whole	−24.6	5.6	12.3	5.6	16.6	7.2	10.0		5.9	n.d.	20.0	9.7	1.7	3.5
Plant																
Brassica oleracea (#1)[c]		Leaf	−32.5	4.7	0.2	−6.7	5.1	3.8	3.9	9.5	1.0	0.8	5.7	13.1	1.4	1.1
Brassica oleracea (#2)		Leaf	n.d.	n.d.	1.7	−7.0	3.7	0.7	3.4	4.6	−2.1	n.d.	2.6	9.8	n.d.	1.2
Brassica oleracea (#3)		Leaf	n.d	n.d.	4.3	−8.0	3.4	−1.2	−0.8	6.3	−5.5	n.d.	3.4	11.7	n.d.	1.0
Daucus carota		Leaf	−30.6	5.9	8.3	−2.2	8.0	4.6	7.3	7.2	n.d.	n.d.	8.2	15.7	1.8	1.1
Castanea crenata (#1)[c]		Leaf	−30.0	0.7	−2.1	−12.7	−0.3	0.5	1.6	4.6	−4.2	n.d.	1.5	10.1	0.2	1.0
Castanea crenata (#2)		Leaf	−29.3	1.8	0.7	−9.8	0.2	−2.4	−0.7	6.2	n.d.	n.d.	2.9	8.8	0.5	1.3
Castanea crenata (#3)[c]		Nut	n.d.	n.d	n.d.	−14.8	n.d.	0.1	n.d.	2.9	−7.2		0.8	8.3	n.d.	1.1
Citrus unshiu		Leaf	−30.6	4.9	6.4	−6.8	4.7	3.1	3.0	8.9	−0.6	n.d.	2.8	12.4	1.5	0.8
Raphanus sativus		Leaf	−29.8	4.0	−3.6	−8.4	−2.9	−3.8	−4.3	3.3	−4.9	n.d.	−2.6	5.9	1.2	1.0
Solanum lycopersicum		Leaf	−28.5	5.2	6.2	−3.6	2.0	2.9	1.3	8.6	−4.0	n.d.	2.0	10.3	1.5	1.0
Solanum melongena		Leaf	−27.7	5.6	5.6	−4.6	6.8	1.3	−0.2	15.1	5.1	n.d.	7.2	17.0	1.7	0.8
Solanum tuberosum		Leaf	−29.5	−2.8	−2.4	−12.1	−2.0	−5.7	−3.5	−2.0	n.d.	n.d.	−6.3	4.1	−0.8	0.7
Catapillar																
Pieris rapae (#1)[c]	Larva	Whole	−29.6	1.9	6.6	−0.4	8.2	7.0	8.9	16.3	3.7	1.6	13.0	13.4	0.6	2.0
Pieris rapae (#2)[c]	Larva	Whole	−26.9	1.9	5.3	−2.7	7.4	6.5	8.5	14.3	2.3	1.0	14.6	13.6	0.6	2.2
Pieris rapae (#3)	Larva	Whole	n.d.	n.d.	9.1	0.1	9.6	2.7	7.0	13.1	1.6	n.d.	10.6	9.5	n.d.	2.2
Pieris rapae (#4)	Larva	Whole	n.d.	n.d.	8.3	0.8	6.5	2.4	4.6	11.8	1.5	n.d.	9.9	11.9	n.d.	1.8
Aphid																
Aphidoidea sp.	Adult	Whole	−21.8	2.2	5.5	2.9	7.1	3.6	5.1	10.4	1.2	n.d.	8.1	8.9	0.7	2.0
Ladybug																
Harmonia axyridis	Larva	Whole	−26.5	6.4	10.8	8.4	9.4	4.9	9.1	18.7	3.7	n.d.	16.5	9.0	1.9	3.1
Harmonia axyridis	Chrysalis	Whole	−29.0	4.7	10.2	7.9	10.1	5.0	7.8	15.0	3.7	n.d.	16.6	9.4	1.4	3.1
Harmonia axyridis	Adult	Whole	−28.6	7.4	10.9	11.7	9.8	7.4	8.8	23.0	6.6	n.d.	14.2	6.9	2.2	3.1

[a]Trophic position estimated by the traditional bulk isotope method with the following equation: $TP_{Bulk} = (\delta^{15}N_{sample} - \delta^{15}N_{average\ of\ plants})/3.4 + 1$; Minagawa and Wada 1984
[b]Trophic position calculated using the novel amino method with following equation: $TL_{Glu/Phe} = (\delta^{15}N_{Glu} - \delta^{15}N_{Phe} + 8.4)/7.6 + 1$; Chikaraishi et al. 2010a
[c]Data from Chikaraishi et al. 2010a

mandarinia japonica, *Vespa simillima xanthoptera*, and *Vespula flaviceps lewisii*) were collected from the farm. For wasps, we also collected different growth stages of samples: egg, larva, chrysalis, and wasps newly emerged from their nests. Leaves from several plants: *Brassica oleracea* (broccoli), *Daucus carota* (carrot), *Castanea crenata* (chestnut), *Citrus unshiu* (orange), *Raphanus sativus* (radish), *Solanum lycopersicum* (tomato), *Solanum melongena* (eggplant), and *Solanum tuberosum* (potato), nuts from *C. crenata*., and caterpillars, *Pieris rapae*, found on the leaves of *B. oleracea* were also collected as reference materials for primary producers and baseline consumers at this farm. Samples of the aphid *Aphidoidea sp*. and the ladybug *Harmonia axyridis* were also collected from the *C. crenata* leaves, as examples of insect samples with well-established trophic positions (i.e., 2.0 for aphids and 3.0 for ladybugs); aphids feed on the sap from *C. crenata* and ladybugs feed on the aphids.

Sample surfaces were washed with distilled water to remove contaminants. Samples were then freeze-dried and crushed to a fine powder. Powdered samples were stored at −20°C prior to analysis. The carbon and nitrogen isotopic compositions of the bulk sample materials were determined using a Thermo Fisher Scientific Flash EA (EA1112) coupled to a Delta^{plus}XP IRMS with a ConFlo III interface (Ogawa et al. 2010). Carbon and nitrogen isotopic compositions are reported in the standard delta (δ) notation relative to the Vienna Pee Dee Belemnite standard (VPDB) and to atmospheric nitrogen (Air), respectively.

All samples were prepared for CSIA of amino acids with HCl hydrolysis followed by *N*-pivaloyl/isopropyl (Pv/iPr) derivatization, according to the methods described by Chikaraishi et al. (2007). In brief, samples were hydrolyzed using 12 M HCl at 100°C. The hydrolysate was washed with *n*-hexane/dichloromethane (6:5, v/v) to remove hydrophobic constituents (e.g., lipids). Then, derivatizations were performed sequentially with thionyl chloride/2-propanol (1:4, v/v) and pivaloyl chloride/dichloromethane (1:4, v/v). The Pv/iPr derivatives of the amino acids were extracted with *n*-hexane/dichloromethane (6:5, v/v). The nitrogen isotopic compositions of individual amino acids were determined by GC/C/IRMS using an Agilent Technologies 6890N GC coupled to a Thermo Fisher Scientific Delta^{plus}XP IRMS with a GC-C/TC III interface. The analytical conditions for the GC/C/IRMS analyses are described in detail by Chikaraishi et al. (2008, 2010a). Standard mixtures of ten amino acids (alanine, glycine, valine, leucine, norleucine, aspartic acid, serine, glutamic acid, phenylalanine, and hydro) with known δ^{15}N values (ranging from −7.3 to +22.7‰) were analyzed after every three to four samples during GC/C/IRMS analytical sessions to assess the reproducibility of the isotope measurements and to normalize the δ^{15}N values of amino acids in samples. Three pulses of reference N_2 gas (−4.3‰) were discharged into the IRMS at the beginning and end of each chromatogram for both standard mixtures and samples. The isotopic compositions of amino acids in samples are expressed relative to atmospheric N_2 on scales normalized to the known δ^{15}N values of standard amino acids.

Nitrogen isotopic compositions (δ^{15}N) were determined for the following ten amino acids in samples: alanine, glycine, valine, leucine, isoleucine, proline, serine, methionine, glutamic acid, and phenylalanine. These amino acids were chosen because their peaks were always well separated with baseline resolution in the chromatogram (Metges et al. 1996; Chikaraishi et al. 2010b). The accuracy obtained for the standards and samples was always better than ±0.5‰ for sample sizes of ≥30 ng N. Glutamine was converted to glutamic acid during acid hydrolysis; as a result, the α-amino group of glutamine contributed to the δ^{15}N value calculated for glutamic acid. Additional amino acids were not included in the study due to difficulties associated with sample preparation; primarily, the co-elution of a portion of aspartic acid with threonine during chromatography and the absence of arginine, cysteine, histidine, lysine, tyrosine, and tryptophan from chromatograms, which is attributed to the decomposition of samples during preparation or low sample recovery (Chikaraishi et al. 2010b). The trophic position of samples was calculated from the δ^{15}N values for glutamic acid and phenylalanine using Eq. (1) and a β value of +8.4‰.

Results and discussion

δ^{13}C and δ^{15}N values of bulk tissues

The carbon and nitrogen isotopic compositions determined for the bulk tissues of the bees, wasps, and hornets are listed in Table 1, together with those of reference materials (plants, caterpillars, aphids, and ladybugs) collected from the same farm. The δ^{13}C values of bulk tissues are between −26.8 and −25.0‰ for bees, −30.1 and −26.1‰ for wasps, and −25.8 and −24.6‰ for hornets. These δ^{13}C values are similar to or slightly more positive than those determined for plants (between −32.5 and −27.7‰), caterpillars (between −29.6 and −26.9‰), aphids (−21.8), and ladybugs (between −29.0 and −26.5‰). These results are consistent with the previously observed δ^{13}C shift in food webs (−0.6 to +2.7‰ at each trophic level; e.g., DeNiro and Epstein 1981). Thus, the examined insects are presumed to feed entirely on plant materials or consumers in C3 food webs.

Field observations reveal that bee species (i.e., *A. mellifera*, *B. diversus*, and *X. appendiculata*) feed on nectar and pollen from flowers; wasp species (i.e., *P. indica*, *P. japonicus*, and *P. rothneyi*) feed mainly on caterpillars found on plant leaves; the hornet species *V. ducalis* feeds solely on wasps; and other hornet species (i.e., *V. flaviceps*, *V. mandarinia*, and *V. simillima*) are carnivorous predators that feed on various insects, such as caterpillars, bees, and wasps (e.g., Takamizawa 2005). Therefore, the biologically expected trophic positions

are 2.0 for bees, 3.0 for wasps, 4.0 for the hornet species *V. ducalis*, and between 3.0 and 4.0 for the other hornet species. The δ^{15}N values of bulk tissues are between +1.6 and +5.1‰ for bees, between +1.1 and +7.2‰ for wasps, and between +4.5 and +5.6‰ for hornets, which largely overlaps the range of δ^{15}N values for plant materials (between −2.8 and +5.9‰). If an average δ^{15}N value for the examined plant materials is simply employed as a δ^{15}N baseline for the determination of trophic position in the bulk method, trophic positions may be estimated from the δ^{15}N values of bulk tissues (i.e., between 0.5 and 1.5 for bees, between 0.3 and 2.1 for wasps, and between 1.3 and 1.7 for hornets; see Table 1). The estimated trophic positions show only a weak correlation with the expected trophic positions for these insects.

The weak correlation observed between estimated and expected trophic positions is largely attributed to the large variation among the δ^{15}N values exhibited by plants in modern terrestrial environments, especially in the case of a farm. This large variation among δ^{15}N values may also reflect the influence of human activities on terrestrial environments (e.g., Denton et al. 2001; Commisso and Nelson 2006). In fact, in our study, significant variation in δ^{15}N values (ranging from −2.8 to +5.9‰) was observed for bulk plant leaves. This range is equivalent to approximately 2.6 times the ^{15}N-enrichment factor in the bulk method (i.e., 3.4‰; Minagawa and Wada 1984).

Accuracy in CSIA of amino acids

The nitrogen isotopic compositions of ten amino acids in terrestrial insects (bees, wasps, and hornets) and the reference materials (plants, caterpillars, aphids, and ladybugs) are given in Table 1. As with the bulk isotopic compositions, large variability is observed among the δ^{15}N values of amino acids; for phenylalanine, the δ^{15}N values are only weakly increased by trophic level, yet the δ^{15}N values are still highly variable (between +5.7 and +12.9‰ for bees, +3.0 and +14.9‰ for wasps, and +7.7 and +9.7‰ for hornets), likely reflecting large variation among the δ^{15}N values of plants (i.e., the food web base) at this farm.

When CSIA of amino acids is used for plants and insects with well-established trophic positions, the trophic positions estimated from the δ^{15}N values of glutamic acid and phenylalanine are between 1.0 and 1.3 for plants, between 1.8 and 2.2 for aphids, and 3.1 for ladybugs (Fig. 2). The potential uncertainly in the trophic position calculated by taking into account the propagation of uncertainty (i.e., 0.5‰ for $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$, 1.6‰ for β, and 1.2‰ for denominator: Chikaraishi et al. 2010a, b) in Eq. (1) is only 0.23 for plants, 0.28 for aphids, and 0.40 for ladybugs. Thus the trophic positions estimated by CSIA of amino acids are consistent with the actual trophic positions for these plants and insects (1.0 for plants, 2.0 for aphides, and 3.0 for ladybugs). The standard deviation (1σ) on the accuracy (=[actual TP] − [$TP_{Glu/Phe}$]) is 0.11 units for these plants and insects, which is somewhat smaller than that (1σ = 0.20) estimated from plants and caterpillars in our previous study (Chikaraishi et al. 2010a). Combined with the dataset for plants and caterpillars provided by Chikaraishi et al. (2010a), here we employ 0.17 unit (1σ) as the accuracy in the trophic position estimated by CSIA of amino acids for terrestrial C3 food webs.

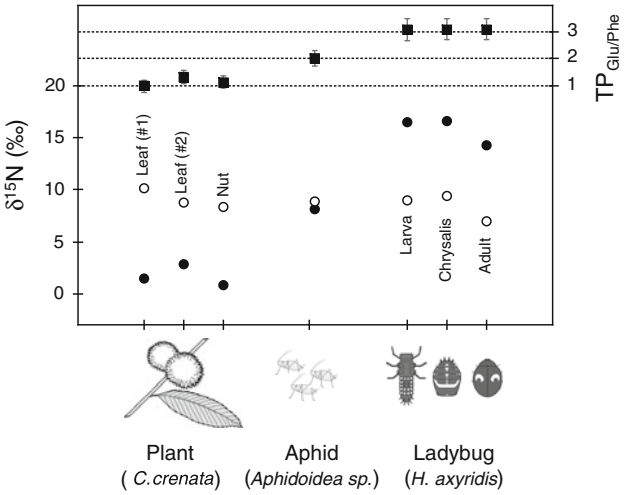

Fig. 2 Nitrogen isotopic compositions of glutamic acid (*filled circles*) and phenylalanine (*open circles*), and trophic positions, estimated by the amino acid method ($TP_{Glu/Phe}$, *filled squares*), for plants (*C. crenata*), aphids (*Aphidea* sp.), and ladybugs (*H. axyridis*). *Bars* represent potential uncertainly in $TP_{Glu/Phe}$ calculated by taking into account the propagation of 1σ for $\delta^{15}N_{Glu}$, $\delta^{15}N_{Phe}$, β, and denominator (7.6) in Eq. (1)

Based on triplicate analysis for representative species, the standard deviation (1σ) for the comparison of trophic positions estimated by CSIA of amino acids is <0.21 (0.13 on average) units for all samples (i.e., plant leaves, caterpillars, bees, wasps and hornets) (Fig. 3a). This variation is almost identical or smaller than the accuracy (1σ = 0.17) of this method. The insects of interest may exhibit large variation among the bulk δ^{15}N values of different body tissues (e.g., leg and wing) because of highly variable compositions of nitrogen-containing molecules, which reflect their feeding habits and life strategies. In fact, the bulk δ^{15}N values for different body tissues of a wasp (*P. rothneyi* #15) exhibit a spread of 2.4‰ among abdomen (+3.8‰), leg and wing (+5.4‰), thorax (+5.7‰), and head (+6.4‰) tissues (Table 1). However, as shown in Fig. 3b, the δ^{15}N values of amino acids, particularly those of glutamic acid and phenylalanine, are within error among tissues (i.e., within 1σ of 0.4‰ for the comparison of the δ^{15}N values). Moreover, the estimated trophic positions are consistent for different tissues (i.e., within 1σ of 0.03 units for the comparison of the $TP_{Glu/Phe}$ values). Thus, no substantial difference is observed in both the δ^{15}N values of amino acids and the estimated trophic positions among different body tissues; e.g., leg and wing.

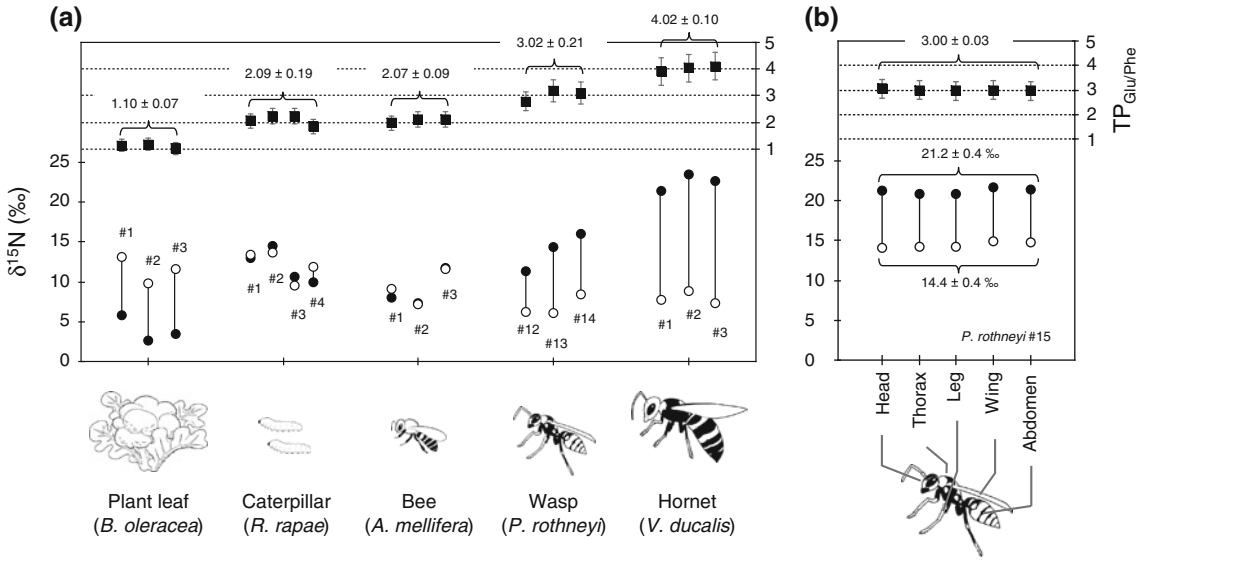

Fig. 3 Nitrogen isotopic compositions of glutamic acid (*filled circles*) and phenylalanine (*open circles*), and trophic positions, estimated by the amino acid method ($TP_{Glu/Phe}$, *filled squares*), for **a** plant (*B. oleracea*), caterpillar (*P. rapae*), bee (*A. mellifera*), wasp (*P. rothneyi*), and hornet (*V. ducalis*) samples (analyzed in triplicate), and **b** different body tissues of a wasp (*P. rothneyi*). *Bars* represent potential uncertainly in $TP_{Glu/Phe}$ calculated by taking into account the propagation of 1σ for $\delta^{15}N_{Glu}$, $\delta^{15}N_{Phe}$, β, and denominator (7.6) in Eq. (1)

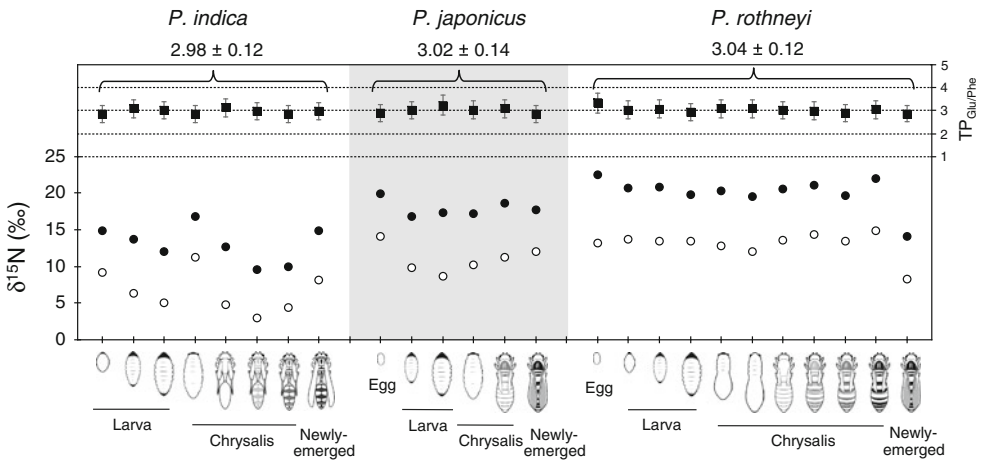

Fig. 4 Nitrogen isotopic compositions of glutamic acid (*filled circles*) and phenylalanine (*open circles*), and trophic positions, estimated by the amino acid method ($TP_{Glu/Phe}$, *filled squares*), for three wasps at various growth stages. *Bars* represent potential uncertainly in $TP_{Glu/Phe}$ calculated by taking into account the propagation of 1σ for $\delta^{15}N_{Glu}$, $\delta^{15}N_{Phe}$, β, and denominator (7.6) in Eq. (1)

Effect of metamorphosis

The lifestyle (e.g., feeding habits) of many insects shows a marked change during metamorphosis from larva to adult. For wasps, after the egg hatches, they feed mainly on caterpillars (hunted by adult worker wasps) during their larva stage, and do not feed during metamorphose in cocoons during their chrysalis stage (e.g., Takamizawa 2005). In a previous study on the Diptera *Chironomus acerbiphilus* (Doi et al. 2007), metamorphosis resulted in an increase of between 0.7 and 1.6‰ in $\delta^{15}N$ values of bulk tissues from larva to adult. Similar increases in $\delta^{15}N$ values have been observed for Lepidoptera (McCutchan et al. 2003; Tabbets et al. 2008) and Neuroptera (Patt et al. 2003).

However, as shown in Fig, 4, our results demonstrate that for wasps (*P. indica*, *P japonicus*, and *P. rothney*), trophic position does not change significantly ($1\sigma < 0.14$ units for the comparison of the $TP_{Glu/Phe}$ values) from the egg to adult stages, despite marked differences among the $\delta^{15}N$ values for wasps at different growth stages (e.g., for phenylalanine, between +3.0 and +11.2‰ for *P indica*, between +8.7 and +14.1‰ for *P japonicus*, and between +8.3 and +14.9‰ for *P rothneyi*). These results suggest that amino acids are not metabolized as energy sources during wasp metamorphosis in cocoons (it could be considered as a semi-closed system), and that metamorphosis has a negligible effect on the $\delta^{15}N$ values of amino acids and the trophic positions of wasps. Rather, the observed variations in

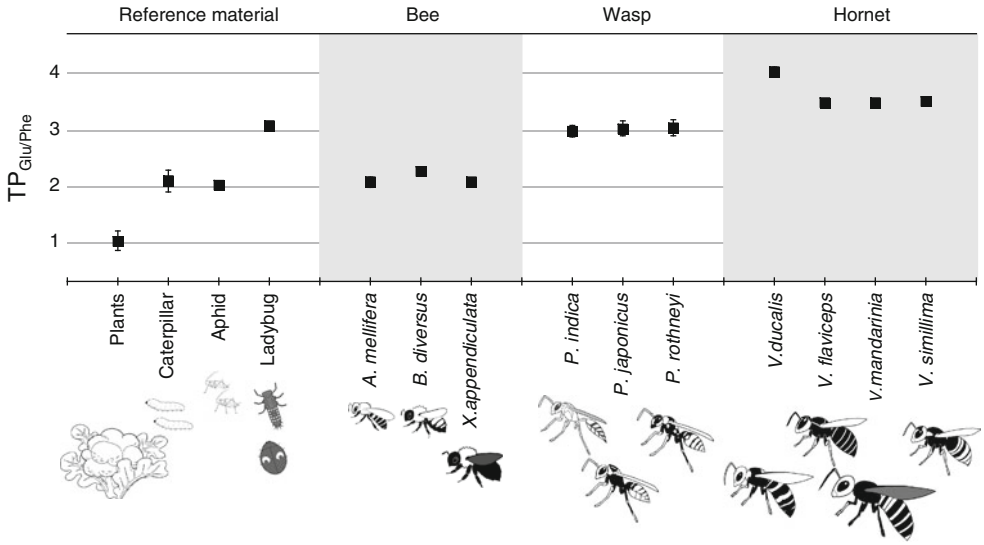

Fig. 5 Mean trophic positions, estimated by the amino acid method, for bees, wasps, hornets, and several reference materials (i.e., plants, caterpillars, aphids, and ladybugs). *Bar* represents 1σ for the comparison of the $TP_{Glu/Phe}$ values in each species

$\delta^{15}N$ values for both bulk tissues and amino acids may reflect changes (or variations) in the $\delta^{15}N$ values of their diet as they feed during the larva stage.

Trophic positions of bees, wasps, and hornets

The bulk $\delta^{15}N$ values vary significantly for plants (between -2.8 and $+5.9‰$) and insects (between $+1.1$ and $+7.2‰$) collected from the farm. Trophic positions estimated by the bulk method have only a weak correlation with the biologically expected trophic positions of these insects (Table 1). However, the trophic positions estimated by CSIA of amino acids are 2.0 and 2.1 for *A. mellifera*, 2.3 for *B. diversus*, 2.1 for *X. appendiculata*, between 2.8 and 3.1 for *P. indica*, between 2.9 and 3.2 for *P. japonicus*, and between 2.8 and 3.3 for *P. rothneyi* (Fig. 5). Considering the accuracy associated with this method ($1\sigma = 0.17$), the estimated trophic positions are well consistent with the biologically expected trophic positions of the bees that feed on nectar and pollen from flowers and of the wasps that feed primarily on caterpillars found on plant leaves. Moreover, the trophic positions estimated for hornets by CSIA of amino acids (between 3.9 and 4.0 for *V. ducalis* and 3.5 for the other hornets, i.e., *V. flaviceps*, *V. mandarinia*, and *V. simillima*; Fig. 5), are consistent with the biologically expected trophic positions for both hornets that feed solely on wasps and other hornets that feed on various insects (e.g., caterpillars, bees, and wasps), respectively.

Herbivorous insects feed on various types of plant tissues such as leaves, twigs, bark, and nectar. In the present study, bees feed on nectar and pollen, caterpillars feed on leaves, and aphids feed on sap. The trophic positions of these insects are estimated to be between 1.8 and 2.3 (2.1 on average), which are reasonable values for the herbivores. Our study suggests that variation among food sources (i.e., plant tissues) does not significantly affect estimates of trophic positions for herbivores. Adult wasps feed on the excretion of larvae as well as nectars from flowers (e.g., Takamizawa 2005), as a result, the trophic positions of adult wasps may be expected to be different from those of larvae and chrysalis. However, no substantial difference is observed among the trophic positions of wasps at different growth stages (i.e., egg, larva, chrysalis, and even adult). This indicates that feeding on excretions and nectars during the adult stages has little effect on the trophic position of adult wasps.

The trophic position of omnivore hornets (*V. flaviceps*, *V. mandarinia*, and *V. simillima*) is estimated to be 3.5. Bulk $\delta^{15}N$ values have been used to estimate trophic positions; however, this is particularly difficult in the case of omnivores. If an omnivore assimilates various food sources with different C:N ratios (i.e., plant and animal), analysis with a simple model using bulk $\delta^{15}N$ values would fail to incorporate differences in C:N stoichiometry among food sources (Post 2002). Using CSIA of amino acids, the trophic position of an omnivore may be estimated using a simple model that considers differences in food quality, because amino acids are assimilated independently of the elemental stoichiometry.

Conclusions

We evaluated the potential of CSIA of amino acids for estimating the trophic position of terrestrial insects (i.e., bees, wasps, and hornets) in a case study of terrestrial food webs. The CSIA estimated trophic positions (yielding values of between 2.0 and 2.3 for bees, between 2.8 and 3.3 for wasps, and between 3.5 and 4.1 for hornets) are well consistent with the biologically expected trophic positions of these insects (i.e., 2.0 for bees, 3.0 for wasps, and 3.0–4.0 for hornets) given the accuracy associated with this method ($1\sigma = 0.17$). In addition, we determined that wasp metamorphosis may

not significantly affect the $\delta^{15}N$ values of amino acids as well as trophic position. Thus, estimates of trophic position based on CSIA of amino acids are useful in investigations of terrestrial food webs with predatory insects.

Our results suggest that CSIA of amino acids is a useful tool for assessing the structure of food webs and biogeochemical processes in both aquatic and terrestrial environments. However, the present method cannot be applied directly to a more complex food web, where the basal resources include terrestrial C3 and C4 plants, as well as aquatic plants, because the factor β in Eq. (1) is different among the basal resources (Fig. 1). In such cases, the mixing ratio among different resources should be quantified by other proxies prior to estimating trophic position. We suggest the use of carbon isotope analysis of bulk tissues or amino acids to address this problem. Advances in this field are expected from recent developments and improvements in methodologies for CSIA of amino acids using $\delta^{13}C$ (Corr et al. 2007a, b; Smith et al. 2009; Chikaraishi and Ohkouchi 2010; Choy et al. 2010). CSIA of amino acids, using both $\delta^{13}C$ and $\delta^{15}N$ values, would facilitate the study of ecological food webs where C3, C4, and aquatic plants are present.

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